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REMARKS

Entry of the foregoing and further and favorable reconsideration of the subject application in light of the following remarks, pursuant to and consistent with 37 C.F.R. § 1.112, are respectfully requested.

By the foregoing amendment, claims 1-11, 13-16, and 18-20 have been canceled without prejudice or disclaimer of the subject matter recited therein. Further, claim 12 has been amended to further clarify Applicants' invention, and cancelled claims 2-6, 8-10 and 20 have been rewritten as new claims 21-29, which depend from allowable claim 17. No new matter has been added.

I. Rejections Under 35 U.S.C. § 112

Claims 13 and 14 have been rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification. This rejection has been rendered moot in view of the cancellation of claims 13 and 14.

Claim 15 has been rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite. This rejection has been rendered moot in view of the cancellation of claim 15.

Accordingly, applicants respectfully request withdrawal of the § 112 rejections.

II. Rejections Under 35 U.S.C. § 103

Claims 1-11 have been rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Japanese abstract 7196523. This rejection has been rendered moot in view of the cancellation of claims 1-11. However to the extent that this rejection applies to new claims 21-29, it is respectfully traversed.

New claims 21-29 depend from claim 17, which the Examiner has deemed allowable (see page 5 of the Office Action). Dependent claims are non-obvious under 35 U.S.C. § 103 if the independent claim(s) from which they depend is non-obvious. *In re Fine*, 837 F.2d 1071, 1076 (Fed. Cir. 1988). Therefore, new claims 21-29 are not obvious over Japanese abstract 7196523.

Claim 12 has been rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Japanese abstract 19940012143. Applicants respectfully traverse this rejection.

Japanese abstract 19940012143 relates to a solution for supplying metallic ions that are deficient in a human subject and simultaneously promoting metabolic functions in the liver to promote health.

This abstract does not teach or suggest a method of maintaining long biological activity and high concentration of ascorbate and a derivative of quercetin in a human comprising orally administering a composition comprising (a) ascorbic acid, ascorbate or a derivative thereof, in combination with (b) one or more of quercetin-3-O-glucoside (isoquercetin), quercetin-4'-glucoside, quercetin-3'-glucoside, or quercetin-7-glucoside, in a molar ratio of from about 2:1 to about 1:2, the amounts being sufficient that the periods of biological activity of (a) and (b) are longer than the periods of biological effectiveness of (a) administered without (b) and of (b) administered without (a). (For a better understanding of the biochemical mechanism behind the method of extending the biological life of ascorbic acid, ascorbate or derivatives thereof, applicants submit herewith non-prior art Song et al., *J. Biol. Chem.*, 277(18):15252-60 (2002).)

Japanese abstract 19940012143 does not teach or suggest the claimed molar ratio range from 2:1 to 1:2 or that the amounts of ascorbic acid and quercetin glucoside present are such that the biological effectiveness of both compounds is maintained. Therefore, claim 12 is not obvious over Japanese Patent Abstract 19940012143.

The Examiner has stated that the cited abstract teaches the use of the claimed flavonoid glycosides in combination with vitamin C and ions for promoting the metabolic function of the body. Assuming, *arguendo*, that the Examiner's statement is correct, this still does not suggest or teach the claimed invention as discussed above.

Claims 13 and 15 have been rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Japanese abstract 6199693. This rejection has been rendered moot in view of the cancellation of claims 13 and 15.

Claims 14 and 18 have been rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Virijesen et al. This rejection has been rendered moot in view of the cancellation of claims 14 and 18.

Claims 19 and 20 have been rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Japanese abstract 04099771. This rejection has been rendered moot in view of the cancellation of claims 19 and 20. However to the extent that this rejection applies to new claim 21 (old claim 20), it is respectfully traversed.

New claim 21 depends from claim 17, which the Examiner has deemed allowable (see page 5 of the Office Action). Dependent claims are non-obvious under 35 U.S.C. § 103 if the independent claim(s) from which they depend is non-obvious. *In re Fine*, 837 F.2d 1071, 1076 (Fed. Cir. 1988). Therefore, new claim 21 is not obvious over Japanese abstract 04099771.

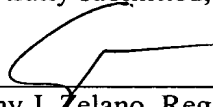
Accordingly, applicants respectfully request withdrawal of the § 103 rejections.

In view of the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order. Such action is earnestly solicited.

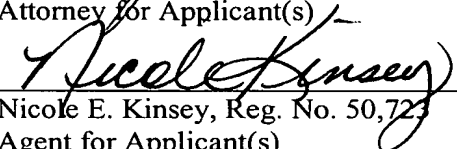
In the event that there are any questions relating to this application, it would be appreciated if the Examiner would telephone the undersigned attorney or agent concerning such questions so that prosecution of this application may be expedited.

The Commissioner is hereby authorized to charge any fees associated with this response or credit any overpayment to Deposit Account No. 13-3402.

Respectfully submitted,



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Flavonoid Inhibition of Sodium-dependent Vitamin C Transporter 1 (SVCT1) and Glucose Transporter Isoform 2 (GLUT2), Intestinal Transporters for Vitamin C and Glucose*

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Vitamin C and flavonoids, polyphenols with uncertain function, are abundant in fruits and vegetables. We postulated that flavonoids have a novel regulatory action of delaying or inhibiting absorption of vitamin C and glucose, which are structurally similar. From six structural classes of flavonoids, at least 12 compounds were chosen for studies. We investigated the effects of selected flavonoids on the intestinal vitamin C transporter SVCT1(h) by transfecting and overexpressing SVCT1(h) in Chinese hamster ovary cells. Flavonoids reversibly inhibited vitamin C transport in transfected cells with IC_{50} values of 10–50 μ M, concentrations expected to have physiologic consequences. The most potent inhibitor class was flavonols, of which quercetin is most abundant in foods. Because Chinese hamster ovary cells have endogenous vitamin C transport, we expressed SVCT1(h) in *Xenopus laevis* oocytes to study the mechanism of transport inhibition. Quercetin was a reversible and non-competitive inhibitor of ascorbate transport; K_i 17.8 μ M. Quercetin was a potent non-competitive inhibitor of GLUT2 expressed in *Xenopus* oocytes; K_i 22.8 μ M. When diabetic rats were administered glucose with quercetin, hyperglycemia was significantly decreased compared with administration of glucose alone. Quercetin also significantly decreased ascorbate absorption in normal rats given ascorbate plus quercetin compared with rats given ascorbate alone. Quercetin was a specific transport inhibitor, because it did not inhibit intestinal sugar transporters GLUT5 and SGLT1 that were injected and expressed in *Xenopus* oocytes. Quercetin inhibited but was not transported by SVCT1(h). Considered together, these data show that flavonoids modulate vitamin C and glucose transport by their respective intestinal transporters and suggest a new function for flavonoids.

Flavonoids are polyphenols that are widely distributed in plant foods and ingested by humans. Flavonoids are subdivided into six structural classes, flavones, flavonols, flavanones, isoflavones, anthocyanidins, and catechins. Although some flavonoids have been proposed to be antioxidants, flavonoid function *in vivo* is uncertain (1, 2).

The flavonoid-like compound phloretin was utilized more than four decades ago to inhibit sodium-independent glucose transport (3). Structural analogs of phloretin from the flavonoid classes of flavanones and flavones inhibited sodium-independent glucose efflux from intestinal cells but not sodium-dependent glucose uptake (4). Because glucose is structurally similar to ascorbic acid (ascorbate, vitamin C) and especially to its oxidized product dehydroascorbic acid (5–7), more recent reports describe effects of flavonoids on ascorbate, dehydroascorbic acid, and glucose transport. The flavonol quercetin and the isoflavone genistein at relatively high concentrations of 100 μ M decreased ascorbate transport in three intestinal cell lines (8). The isoflavone genistein but not the related isoflavone daidzein inhibited glucose and dehydroascorbic acid transport in leukemic (HL-60) cells (9). Inhibition of glucose transport by genistein was competitive and occurred in cells overexpressing GLUT1.¹ In other experiments several flavonoid classes inhibited transport of glucose, dehydroascorbic acid, and ascorbate in three leukemic cell types, the most potent inhibition occurring with flavonols, and the effects could not be explained by ascorbate oxidation (10, 11). Glucose and dehydroascorbic acid transport were inhibited competitively, and ascorbate transport was inhibited non-competitively.

Some aspects of the reported effects of flavonoids on transport inhibition are generally consistent given the structural similarities of glucose, dehydroascorbic acid, and ascorbic acid. However, some reported findings are surprising because of the distinct transport mechanisms for these substrates. Glucose is transported by facilitated sodium-independent glucose transporters GLUT1-GLUT4, GLUT6, GLUT8 and by sodium-dependent transporters SGLT1 and SGLT2 (12–18). Dehydroascorbic acid transport is sodium-independent and is mediated by only GLUT1, GLUT3, and GLUT4 (19–21). No glucose transporters transport ascorbate (20). Ascorbate transport is sodium-dependent and is mediated by ascorbate transporters SVCT1 and SVCT2, neither of which transport glucose and dehydroascorbic acid (22, 23).

By coupling these transport mechanisms and the observations that flavonoids inhibited several distinct cellular transport activities, new insights into flavonoid function become possible. The physiologic implications of the inhibitory effects of flavonoids on transport were not previously apparent. This is because inhibitory effects of flavonoids on cellular transport

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¹ The abbreviations used are: GLUT, glucose transporter isoform; CHO, Chinese hamster ovary; HPLC, high performance liquid chromatography; SGLT, sodium-dependent glucose transporter; SVCT1(h), sodium-dependent vitamin C transporter 1, human; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

occurred at concentrations of 15–100 μM (8–11), but peak concentrations of flavonoids in human plasma are $\sim 1 \mu\text{M}$ after flavonoid ingestion (24, 25). We recognized, however, that flavonoid concentrations that inhibit glucose, ascorbate, and dehydroascorbic acid transport are expected in the intestinal lumen (24, 26). We hypothesized that novel functions of flavonoids may be to distribute nutrient absorption throughout small intestine or to frankly inhibit absorption (11).

Because flavonoids inhibited transport of ascorbate, dehydroascorbic acid, and glucose, the available data are consistent with the possibility that flavonoids modulate some intestinal transporters (10, 11). Nevertheless, more compelling evidence for this hypothesis is lacking. For ascorbate, the intestinal ascorbate transporter is SVCT1, but some flavonoid effects were characterized using cells that express SVCT2(11). In experiments with intestinal cell lines, it was not certain whether flavonoid effects were because of inhibition of dehydroascorbic acid transport or ascorbate transport or simply to ascorbate loss (8). For glucose the intestinal transporters are GLUT2 and SGLT1(12, 27), and the GLUT isoform GLUT5 transports fructose only (12), but these enteric transporters were not evaluated for flavonoid effects (9, 10).

To address our hypothesis, in this paper we tested the effects of flavonoids on the ascorbate intestinal transporter SVCT1(h) and the sugar intestinal transporters GLUT2, GLUT5, and SGLT1. The data indicate that flavonols, a flavonoid class abundant in plant foods consumed by humans, are potent non-competitive and reversible inhibitors of SVCT1(h) and GLUT2 at concentrations predicted from dietary ingestion.

EXPERIMENTAL PROCEDURES

Reagents—[^{14}C]Ascorbic acid (8 mCi/mmol) 2-[^3H]deoxyglucose (25.5 Ci/mmol), [^{14}C]fructose (300 mCi/mmol), and [^{14}C]glucose (265 mCi/mmol) were purchased from PerkinElmer Life Sciences. [^{14}C]Quercetin (53 mCi/mmol) was purchased from ChemSyn Laboratories (Lenexa, KS). Quercetin, fisetin, myricetin, rutin, gossypin, apigenin, naringenin, naringin, hesperetin, genistein, luteolin, daidzein, epicatechin, and catechin were purchased from Sigma. Cyanidin, gossypetin, and delphinidin were obtained from Indofine Chemical Co., Inc. (Somerville, NJ).

Cell Transfection and Culture—CHO cells were obtained from ATCC (Manassas, VA). The transfection construct was made by inserting 1797 base pairs of SVCT1(h) into pcDNA 6/V5-His C vector (Invitrogen) between *HindIII* and *EcoRI* cloning sites, in-frame with the C-terminal peptide. CHO cells were transfected with the construct using LipofectAMINE PLUS kit (Invitrogen). One day before transient transfection, CHO cells growing in Ham's F-12 medium on 60-mm plates were incubated with 0.05% trypsin, EDTA (Invitrogen) for 5 min and counted. 10^7 cells were re-plated on 60-mm plates and achieved 50–90% confluency in 24 h in Ham's F-12 medium with 10% heat-inactivated fetal calf serum. Cells were then washed once in phosphate-buffered saline, and 2 ml of Ham's F-12 medium without serum were added to each 60-mm plate. To prepare transfection mixtures for each plate, 2 μg of DNA in 2 μl of H_2O were diluted into 240 μl of medium without serum and 8 μl of AMINE PLUS reagent. In a second tube, 12 μl of LipofectAMINE PLUS reagent was diluted into 238 μl of medium without serum. The two tubes were incubated for 15 min at room temperature, combined, and incubated for an additional 15 min at room temperature. The combined transfection mixture (500 μl) was added to each plate, which was gently swirled and then incubated at 37 $^\circ\text{C}$, 5% CO_2 atmosphere. After 3 h the medium was replaced with Ham's F-12 medium with 10% fetal bovine serum. Transiently transfected cells were used in experiments in 24–48 h.

The reporter gene V5 in the pcDNA 6/V5-His C vector was used to monitor transfection efficiency in transiently transfected cells. Plated cells 24 h after transfection were fixed for 15 min in 1% freshly prepared paraformaldehyde in phosphate-buffered saline. Cells were washed three times with phosphate-buffered saline, permeabilized for 5 min in 0.2% Triton X-100 in phosphate-buffered saline, and incubated for 30 min in phosphate-buffered saline containing 1% bovine serum albumin (blocking buffer). Cells were then incubated for 1 h with anti-V5 mouse antibody diluted 1:200 in blocking buffer. Cells were washed three times with phosphate-buffered saline and incubated for 1 h with sec-

ondary anti-mouse rabbit antibody conjugated with horseradish peroxidase. Cells were washed again three times with phosphate-buffered saline and incubated for 20 min with 3-amino-9-ethylcarbazole and hydrogen peroxide (AEC + Chromogen System, DAKO, Carpinteria, CA). Cells were rinsed for 5 min with H_2O , and stained cells were counted by microscopy. Using the above conditions, transfection efficiency was 30–85%. Cells with the highest transfection efficiency were used for transport experiments.

The blasticidin resistance site in the pcDNA 6/V5-His C vector was used to develop stably transfected cells. Plated CHO cells transiently transfected with SVCT1(h) as above were maintained in Ham's F-12 medium with 10% fetal calf serum and the antibiotic blasticidin (10 $\mu\text{g}/\text{ml}$) for 48 h. Cells were trypsinized as above and diluted to 1.5 cells/ml medium containing blasticidin. 0.2 ml of this medium were added to each well of 96-well cluster plates. Wells with 1 cell were verified by microscopy, marked, and incubated at 37 $^\circ\text{C}$, 5% CO_2 for 2 weeks in the continuous presence of blasticidin. Single cells that developed into clusters were trypsinized as above, transferred to 50-ml flasks, and grown in Ham's F-12 medium with blasticidin. Cells were tested for ascorbic acid transport activity as described below, and cells with the highest activity were used.

Inhibition of Ascorbic Acid Transport in Transfected CHO Cells—Transiently or stably transfected CHO cells in 24-well plates were washed once and incubated with Krebs buffer 30 mmol/liter HEPES, 130 mmol/liter NaCl, 4 mmol/liter KH_2PO_4 , 1 mmol/liter MgSO_4 , 1 mmol/liter CaCl_2 , pH 7.4. Transport was initiated by adding [^{14}C]ascorbic acid and flavonoids together at the indicated concentrations for the times specified. Flavonoids were diluted 1:100 from concentrated stock solutions prepared fresh by dissolving flavonoids in Me_2SO . Control cells were incubated with [^{14}C]ascorbic acid and 1% Me_2SO without flavonoids. After incubation at 37 $^\circ\text{C}$, uptake was stopped by washing cells in ice-cold phosphate-buffered saline. Cells were solubilized in PBS containing NaOH (0.1 mol/liter) and CHAPS (10 g/liter; J. T. Baker Inc.) and analyzed by scintillation spectrometry or HPLC as described (28, 29). Data displayed represent mean values \pm S.D. of 3 replicates, and each experiment was repeated a minimum of 3 times with similar results. Error bars were omitted when the S.D. was less than symbol size.

Inhibition of Ascorbic Acid and Glucose Transport in *Xenopus laevis* Oocytes—*X. laevis* oocytes were isolated and injected with cRNA coding for the ascorbic acid transporter SVCT1(h) or glucose transporters GLUT2, GLUT5, or SGLT1 as described (20, 23). Briefly, ovaries were resected from adult female frogs anesthetized with 3-aminobenzoic acid ethyl ester (2 g/750 ml) (Sigma) in ice water. Ovarian lobes were opened and incubated in 2 changes of OR-2 without calcium (5 mM HEPES, 82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl_2 , 1 mM Na_2HPO_4 , 100 $\mu\text{g}/\text{ml}$ gentamicin, pH 7.8) with collagenase (2 mg/ml) (Sigma) for 30 min each at 23 $^\circ\text{C}$. Individual oocytes (stages V and VI) were isolated from connective tissue and vasculature, transferred to calcium-containing OR-2 (1 mM CaCl_2), and maintained at 18–20 $^\circ\text{C}$ until injection with cRNA. Oocytes were injected utilizing a Nanoject II injector (Drummond Scientific, Broomall, PA). Injection volumes were 36.8 nl, and cRNA concentrations 1 ng/nl. Sham-injected oocytes were injected with 36.8 nl of water. Post injection oocytes were maintained at 18–20 $^\circ\text{C}$ until experiments were performed.

Two days post-injection, oocytes were equilibrated at room temperature in OR-2. To begin experiments, flavonoids and [^{14}C]ascorbate or 2-[^3H]deoxyglucose or [^{14}C]fructose or [^{14}C]glucose were added together at the indicated concentrations for the times specified. Flavonoids in Me_2SO were prepared as described for cells. Control oocytes were incubated in substrate with 1% Me_2SO without flavonoids. After incubation at room temperature, oocytes were washed four times with ice-cold phosphate-buffered saline. Individual oocytes per replicate were solubilized with 10% SDS, and internalized radioactivity was quantified by scintillation spectrometry as pmol/oocyte. Each data point represents the mean value of 10–15 oocytes \pm S.D. Each experiment was repeated a minimum of 3 times with similar results. Error bars were omitted when the S.D. was less than symbol size.

Effects of Quercetin in Rats—Effects of quercetin on hyperglycemia were tested using Zucker-fa/fa rats (Harlan Sprague-Dawley, Indianapolis, IN). Rats were fed a diet providing recommended allowances for all nutrients and were housed individually in standard hanging stainless steel cages in an environmentally controlled animal research facility maintained at 25 $^\circ\text{C}$ and 55% relative humidity. For experiments, 5 rats 9–15 weeks old were fasted overnight. The following morning a glucose solution of 2 g/kg of body weight without quercetin was administered by oral drip. Blood samples were collected by tail vein bleeding at 0, 15, 30, 45, and 60 min. Glucose

concentrations were immediately determined by glucometer (Dex, Bayer Corp., Elkhart, IN). The experiment was repeated on the same rats within 5 days using a glucose solution of 2 g/kg of body weight plus quercetin, 3–65 mg/kg of body weight. Results were matched to each rat in the presence and absence of quercetin. Each data point represents the mean of five animals \pm S.E. Glucose with and without quercetin was administered at least four times over 4 weeks to the same rats with similar results, and no animals became overtly ill or hypoglycemic. For time course experiments, the area under the curve was determined for each condition, and the statistical significance was calculated by two-tailed paired *t* test. For dose experiments, data from the 30-min control time point (no quercetin) were normalized to 100%. The effect of each quercetin dose at this time point was expressed as percent after calculating as follows.

$$\frac{\text{Blood glucose 30 min after glucose administration with quercetin} - \text{base-line value}}{\text{Blood glucose 30 min after glucose administration without quercetin} - \text{base-line value}} \quad (\text{Eq. 1})$$

Effects of quercetin on ascorbic acid absorption were studied in normal CD (Sprague-Dawley) rats 12–15 weeks old with implanted carotid artery catheters (Charles River Laboratories, Wilmington, MA). Individual rats were housed and fed as described above. Before the experiments, 10 rats were fasted overnight with access to water. To begin experiments the following day, blood from arterial catheters as a zero time value was drawn immediately before administration of the experimental solution. Experimental solutions were fed by gavage and contained either ascorbic acid alone 60 mg/kg of body weight or ascorbic acid 60 mg/kg of body weight with quercetin 15 mg/kg of body weight. Blood was drawn at 30, 60, 120, 180, and 240 min and placed on ice for processing. The experiment was repeated on the same rats with the alternate solution within 1 week. Arterial catheters were maintained with heparinized solutions of glycerol and NaCl, and blood was withdrawn according to the provider's recommendations. After 100 μ l of blood was withdrawn and discarded, 100 μ l of blood was withdrawn and transferred to heparinized Microtainer tubes (Becton Dickinson, Franklin Lakes, NJ). Catheters were flushed with 100 μ l of heparinized NaCl solution after each blood withdrawal. Ascorbic acid was analyzed as described (30). Each point represents the mean of data from 10 animals \pm S.E. Results were matched to each rat in the presence and absence of quercetin. The area under the curve was determined for each condition, and statistical significance was calculated by two-tailed paired *t* test. Ascorbic acid with or without quercetin was administered at least 3 times over 4 weeks to the same rats with similar results.

RESULTS

Effect of Flavonoids on Ascorbate Transport in SVCT1(h)-transfected Cells—To study the effects of flavonoids on sodium-dependent ascorbate transport, CHO cells were transfected with SVCT1(h). Compared with vector-alone-transfected cells, ascorbate transport increased 5–6-fold over 15 min in cells stably transfected with SVCT1(h) (Fig. 1A). Increased transport was linear for at least 15 min. Results were similar in transiently transfected cells (not shown). Because of their abundance in foods, the flavonoid quercetin and its glycone precursor rutin were tested for their effects on ascorbate transport. Cells stably transfected with SVCT1(h) were incubated with ascorbate 10–400 μ M for 10 min with or without 50 μ M quercetin, 50 μ M rutin, or sodium. Ascorbate transport was inhibited ~80% by quercetin, virtually completely when sodium was replaced by choline, and was unaffected by rutin (Fig. 1B). Again, results were similar in transiently transfected cells (not shown). The findings indicate that the flavonoid quercetin is a potent inhibitor of ascorbate transport by SVCT1(h) in transiently or stably transfected cells. For consistency, stably transfected cells were used to characterize flavonoid effects.

Flavonoids (Fig. 2A) consist of a benzopyran configuration (rings A and C) linked to a benzene ring (ring B). Variations in the heterocyclic C-ring and the linkage between the benzopyran and benzene rings are the basis for classifying flavonoids into six groups (31) (Fig. 2B). Within groups, compounds can also be classified whether they are non-glycosylated (aglycones) or glycosylated (glycones). Flavonoid groups and representative aglycone compounds for each are: flavonol (quercetin, rutin

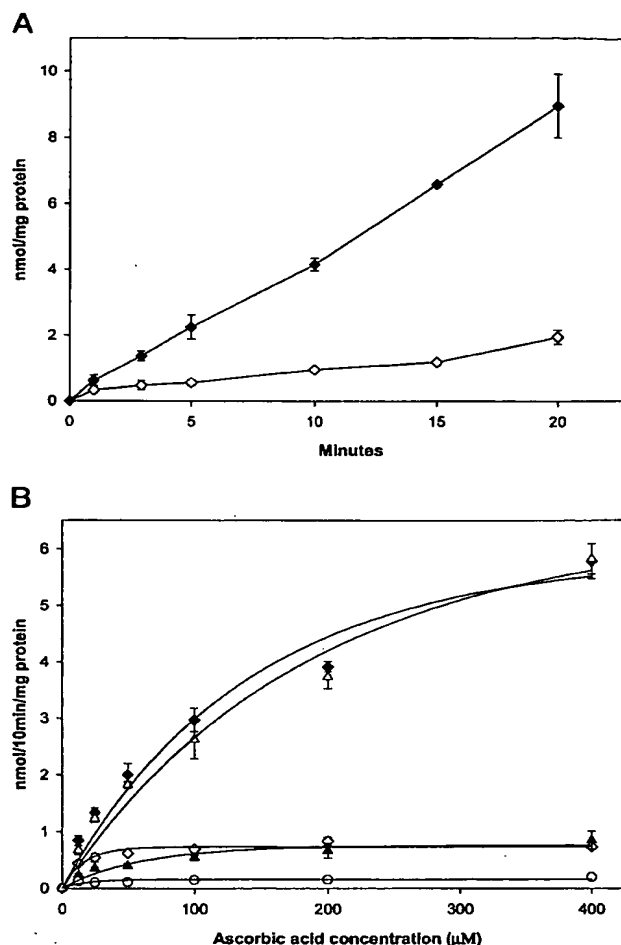


FIG. 1. Time course, concentration dependence, and inhibition of ascorbic acid transport in stably transfected CHO cells. A, time course of ascorbic acid transport in CHO cells stably transfected with SVCT1(h). Confluent pcDNA 6/V5-His C vector (\diamond) and SVCT1(h)-transfected CHO cells (\blacklozenge) were incubated with 300 μ M [14 C]ascorbic acid and 1% Me₂SO for different times at 37 $^{\circ}$ C. Cells were then washed 4 times with 4 $^{\circ}$ C phosphate-buffered saline and solubilized in NaOH (0.1 N), 1% CHAPS, and cell-associated radioactivity was quantified. Data represent the mean \pm S.D. See methods for details. B, concentration dependence and inhibition of ascorbic acid transport in CHO cells stably transfected with SVCT1(h). Transport was initiated by adding [14 C]ascorbic acid at the indicated concentrations with 50 μ M quercetin (\blacktriangle) or 50 μ M rutin (\triangle) or Me₂SO (1%) alone (\blacklozenge). Transport was also measured at the indicated [14 C]ascorbic acid concentrations with 1% Me₂SO in pcDNA 6/V5-His C vector-transfected CHO cells (\diamond) and in SVCT1(h)-transfected CHO cells with choline chloride replacing sodium chloride (\circ). After a 10-min incubation, cells were washed, and transport was quantified as described in A and B and "Experimental Procedures."

(glycone form of quercetin), myricetin, fisetin, gossypetin, gossypin (glycone form of gossypetin); flavone (apigenin); flavanone (naringenin, naringin (glycone form of naringenin)); isoflavone (genistein, daidzein); catechin (catechin); and anthocyanin (cyanidin). There are similarities between the chemical structure of ascorbate and some flavonoids, especially flavonols (Fig. 2C).

Because quercetin was an effective inhibitor and is a flavonol, other flavonols were tested for inhibition of ascorbate transport in cells stably transfected with SVCT1(h). Fisetin, myricetin, and quercetin each had a similar IC₅₀ for transport inhibition (Fig. 3A, Table I). Inhibition was eliminated if gly-

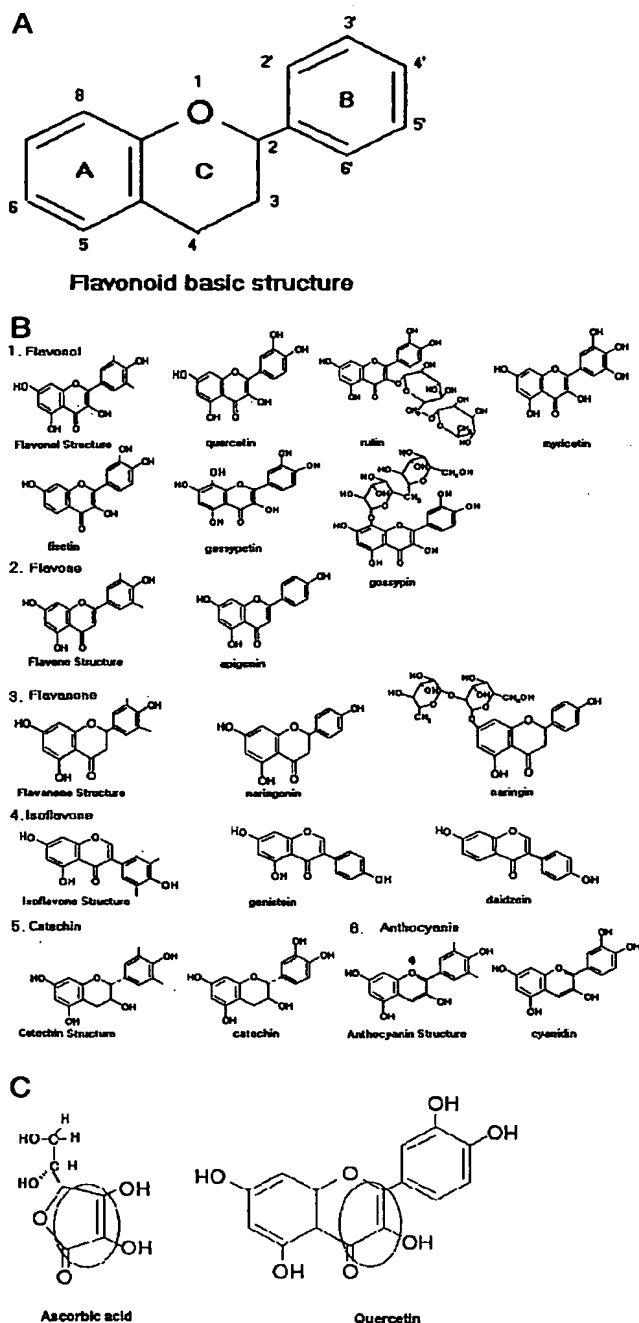


FIG. 2. Flavonoid structure and flavonoid classes. A, flavonoids have benzopyran (rings A and C) and benzene (ring B) moieties. B, different flavonoid classes have variations in the C ring and in the linkage between the benzopyran and benzene rings. Flavonoids can have glycosylated and aglycone forms. Structures of each flavonoid class are followed by examples of that class. See text for details. Flavonols are quercetin, rutin, fisetin, myricetin, gossypetin, and gossypin. Flavones are represented by apigenin. Flavonones are naringenin and naringin. Isoflavones are genistein and daidzein. Catechins are represented by catechin. Anthocyanins are represented by cyanidin. C, structural similarities between quercetin and ascorbic acid are circled.

cosylated residues were present at the 3 or 8 positions (rutin and gossypin, respectively) and when the hydrogen at the 8 position was replaced with a hydroxyl group (gossypetin).

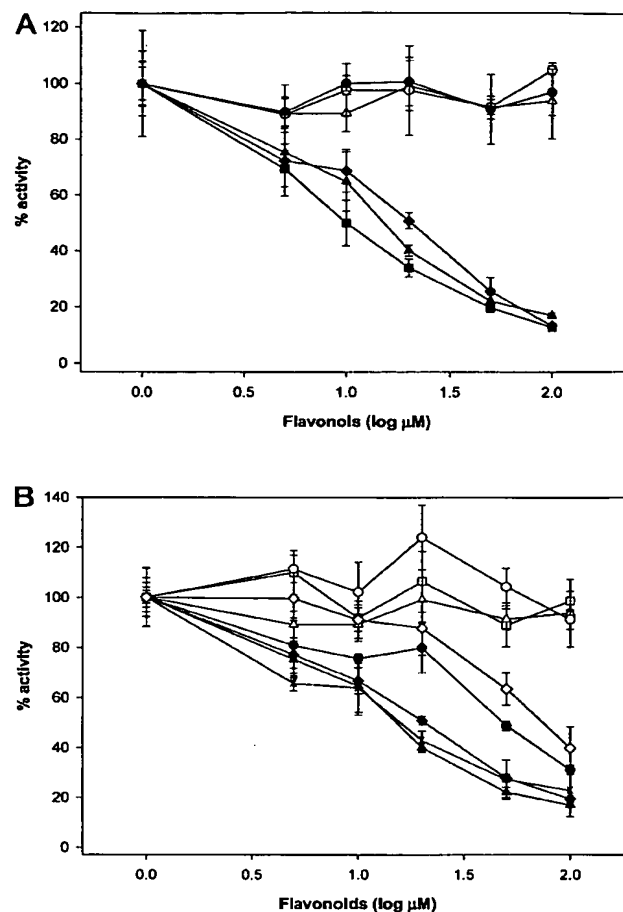


FIG. 3. Flavonoid inhibition of ascorbic acid transport in SVCT1(h) stably transfected cells. CHO cells stably transfected with SVCT1(h) were incubated with the indicated concentrations of flavonoids and [^{14}C]ascorbic acid 300 μM in 1% Me_2SO for 5 min. Cells were washed and transport quantified as in Fig. 1. A, incubation with flavonoids from the flavonol class. Δ , rutin; \blacktriangle , quercetin; \blacksquare , myricetin; \blacklozenge , fisetin; \bullet , gossypetin; \circ , gossypin. B, incubation with compounds from different flavonoid classes. Flavonol class (Δ , quercetin; Δ , rutin); flavone class (\times , apigenin); flavanone class (\bullet , naringenin; \circ , naringin); isoflavone class (\blacklozenge , genistein); catechin class (\square , catechin); anthocyanin class (\diamond , cyanidin).

Compounds in other flavonoid groups were tested for inhibition of transport. The tested compounds in rank of most to least inhibitory were myricetin > quercetin > fisetin = apigenin > genistein > naringenin > cyanidin >>> naringin = catechin = rutin = gossypin = gossypetin (Fig. 3, A and B, Table I). The data in Figs. 2 and 3 suggest that inhibition of SVCT1(h) by flavonoids is affected by the presence of a double bond at C2-C3 and a ketone at C4 and that these moieties confer structural similarity to ascorbate (Fig. 2, A and C). Inhibition of transport was also eliminated by substitution of the hydrogen at C8 and by glycosylation at C3 or C8 (see Fig. 2A).

We tested other possibilities to explain flavonoid inhibition of ascorbate transport. To test reversibility, cells were preincubated with quercetin 100 μM under the conditions that inhibited ascorbate transport, washed, and incubated with ascorbate without quercetin. Inhibition of transport was entirely reversible. Ascorbate transport without quercetin preincubation was 4.4 nmol/10 min/mg of protein and, with quercetin preincubation and washing, was 4.3 nmol/10 min/mg of protein. Without Me_2SO , transport was 4.2 nmol/10 min/mg of protein, indicat-

TABLE I
Flavonoid concentration inhibiting ascorbic acid transport by 50% (IC_{50}) in SVCT1(h)-transfected CHO cells
NI, not inhibitory.

Flavonoids		IC_{50}
		μM
Flavonol	Quercetin	15
	Rutin (glycone of quercetin)	NI
	Fisetin	16
	Myricetin	11
	Gossypetin	NI
Flavone	Gossypin (glycone of gossypetin)	NI
	Apigenin	16
Flavanone	Naringenin	49
	Naringin (glycone of naringenin)	NI
Isoflavone	Genistein	20
Catechin	Catechin	NI
Anthocyanin	Cyanidin	84

ing that Me_2SO alone did not inhibit cellular transport. HPLC ascorbate analyses showed that ascorbic acid concentrations were unchanged in the presence of quercetin 100 μM , indicating that the transport findings could not be explained by ascorbate oxidation by quercetin (32) (not shown).

Effect of Flavonoids on Ascorbic Acid Uptake in SVCT1(h)-injected Oocytes—To determine the kinetics mechanism of ascorbate inhibition of SVCT1(h), it was necessary to utilize a system that expressed only SVCT1(h). CHO cells had basal ascorbate transport (Fig. 1, A and B). Using reverse transcription-PCR, SVCT2 but not SVCT1 was detected in CHO cells (not shown). Endogenous SVCT2 would interfere with accurate determination of inhibition kinetics of flavonoids with respect to SVCT1. Because *X. laevis* oocytes are not expected to transport ascorbate basally (23), we used the oocyte expression system to characterize inhibition kinetics of flavonoids on SVCT1(h).

The time course and concentration dependence of ascorbate transport were determined in *X. laevis* oocytes injected with cRNA for SVCT1(h) (Fig. 4). Ascorbate transport was linear for at least 20 min, and sham-injected oocytes did not transport ascorbate. Using an ascorbate incubation time of 5 min, the K_m of SVCT1(h) was 149 μM , similar to previous values (23).

Oocytes injected with SVCT1(h) were incubated with 300 μM ascorbate for 5 min with or without flavonols 5–100 μM (Fig. 5A). Quercetin and fisetin had an IC_{50} of 32 and 14 μM , respectively, whereas rutin was not inhibitory. Using a fixed flavonoid concentration of 50 μM , we tested the effect of each flavonoid class on ascorbate transport (Fig. 5B). The data show that flavonols were the most potent inhibitors of SVCT1(h) expressed in oocytes. Ascorbate transport in oocytes (Fig. 5B) was inhibited by other flavonoids in a fashion generally consistent with flavonoid inhibition in cells transfected with SVCT1(h) (Fig. 3, A and B).

Reversibility was tested of quercetin inhibition of SVCT1(h) expressed in oocytes. Ascorbate transport with 300 μM ascorbate in 1% Me_2SO was 126 pmol/10 min/oocyte; with 100 μM quercetin preincubation for 10 min and washing followed by 300 μM ascorbate in 1% Me_2SO ascorbate transport was 117 pmol/10 min/oocyte; and with 300 μM ascorbate without Me_2SO or quercetin ascorbate transport was also 117 pmol/10 min/oocyte. These data indicate the quercetin inhibition of SVCT1(h) was reversible and that Me_2SO did not inhibit transport.

Because SVCT1(h) was the only ascorbate transporter injected and expressed in oocytes, and quercetin inhibition was reversible, inhibition kinetics could be determined. Oocytes injected with SVCT1(h) were incubated with 50–800 μM ascorbate and 0–50 μM quercetin for 5 min. Quercetin inhibited

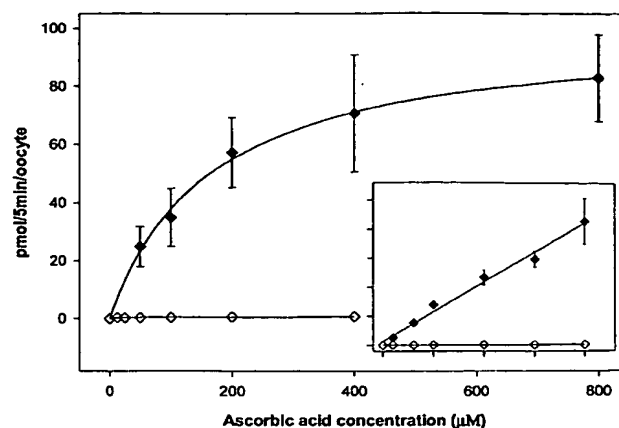


FIG. 4. Concentration dependence of ascorbic acid uptake in SVCT1(h)-injected oocytes. SVCT1(h) cRNA-injected oocytes (\blacklozenge) and sham-injected oocytes (\circ) were incubated with [^{14}C]ascorbate 0–800 μM in 1% Me_2SO for 5 min. Oocytes were washed, and transport was quantified as noted under "Experimental Procedures." Inset, time course of ascorbic acid uptake in SVCT1(h)-injected oocytes. SVCT1(h) cRNA-injected oocytes (\blacklozenge) and sham-injected oocytes (\circ) were incubated for 0–20 min at 23 $^{\circ}C$ with [^{14}C]ascorbic acid 300 μM in 1% Me_2SO . The x axis represents 0–20 min, and the y axis represents 0–250 pmol/oocyte. Oocytes were washed, and transport was quantified as noted under "Experimental Procedures."

ascorbate transport noncompetitively; K_i 17.8 μM (Fig. 6).

We attempted to determine whether SVCT1(h) transported quercetin. Oocytes injected with SVCT1(h) and sham-injected oocytes were incubated with [^{14}C]quercetin (100 μM) to measure uptake. SVCT1(h)-injected oocytes had quercetin uptake of 4.6 pmol/5 min/oocyte, and sham-injected oocytes had quercetin uptake of 4.0 pmol/5 min/oocyte. These data suggest that SVCT1(h) does not transport quercetin.

Effects of the Flavonol Quercetin on Intestinal Glucose Transporters—We investigated whether quercetin inhibited the intestinal glucose transporter GLUT2. Oocytes were injected with GLUT2 and incubated with 2-[3H]deoxyglucose in the presence or absence of quercetin 10 or 20 μM (Fig. 7). Glucose transport was inhibited non-competitively; K_i 22.8 μM . Inhibition of GLUT2-mediated transport activity in oocytes by 100 μM quercetin was reversible (not shown).

The effect of quercetin was tested on the other intestinal sugar transporters GLUT5 and SGLT1. Oocytes injected with GLUT5 were incubated with [^{14}C]fructose (100 μM), the substrate for GLUT5, with or without quercetin 100 μM . Without quercetin, fructose transport was 4.6 pmol/10 min/oocyte, with quercetin, fructose transport was 4.2 pmol/10 min/oocyte, and in sham-injected oocytes, fructose transport was 0.2 pmol/10 min/oocyte. These data indicate that quercetin did not inhibit fructose transport by GLUT5. Similar experiments were performed with SGLT1 expressed in oocytes. Transport of [^{14}C]glucose 1 mM was \sim 200 pmol/10 min/oocyte and was unchanged by quercetin 5–100 μM . Thus, quercetin did not inhibit glucose transport by SGLT1. Taken together, these data suggest that quercetin inhibition of SVCT1 and GLUT2 is not mediated by general nonspecific effects on transmembrane transport proteins.

Effects of Quercetin on Glucose and Ascorbic Acid Absorption in Rats—GLUT 2 has been suggested to be the major intestinal glucose transporter under physiologic conditions (27, 33, 34). To test the physiologic relevance of the effects of quercetin on glucose absorption, the effects of quercetin were tested in diabetic Zucker fa/fa rats. Animals fed glucose without quercetin were hyperglycemic, with plasma glucose values approaching

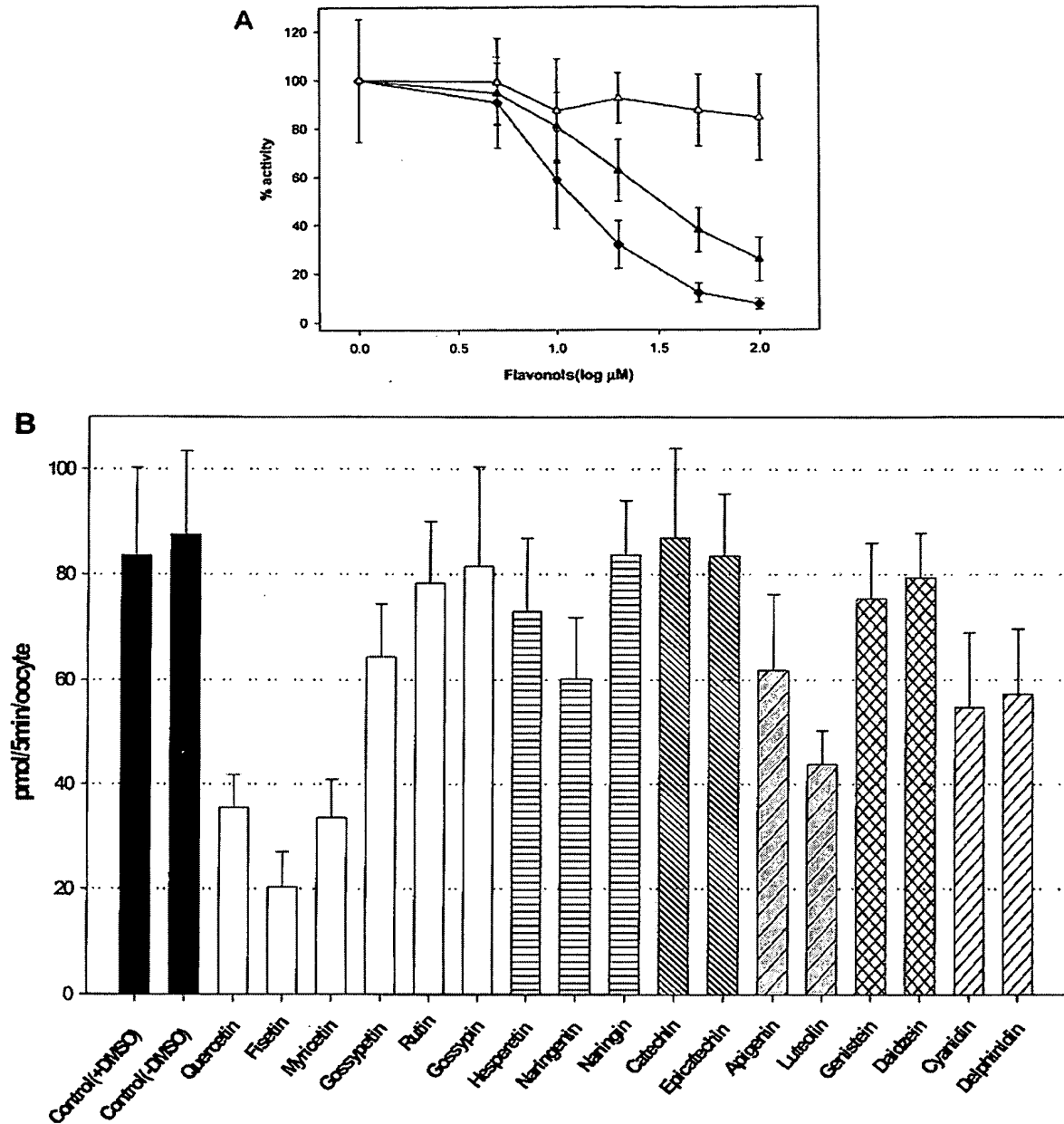


FIG. 5. Flavonoid inhibition of ascorbate transport in SVCT1(h) cRNA-injected oocytes. *A*, injected oocytes were incubated for 5 min with ascorbic acid 300 μM and flavonols 5–100 μM . Controls were incubated with ascorbic acid in buffer and 1% Me₂SO without flavonol. Flavonols used were rutin (Δ), quercetin (\blacktriangle), and fisetin (\blacklozenge). *B*, injected oocytes were incubated for 5 min with 50 μM indicated flavonoid and 300 μM [¹⁴C]ascorbic acid. Oocytes were washed and transport quantified as described under "Experimental Procedures." Controls with 300 μM ascorbic acid with and without 1% Me₂SO (DMSO) are also shown. Bars represent controls (filled bars) and the following flavonoid classes (from left to right): flavonol (quercetin, fisetin, myricetin, gossypetin, rutin, and gossypin) (open bars), flavanone (hesperetin, naringenin, and naringin), catechin (catechin and epicatechin), flavone (apigenin and luteolin), isoflavone (genistein and daidzein), and anthocyanidin (cyanidin and delphinidin).

300 mg/dl 30 min after glucose ingestion (Fig. 8A). When quercetin was ingested with glucose, hyperglycemia was significantly decreased ($p = 0.015$). These data suggest that quercetin might inhibit glucose absorption via GLUT2 *in vivo*. The effect of quercetin on hyperglycemia was dose-dependent (Fig. 8B).

Ascorbate absorption from the gastrointestinal tract is difficult to demonstrate in experimental animals (35, 36). One explanation is that during blood procurement hemolysis occurs, resulting in ascorbate oxidation. To address this prob-

lem, absorption experiments were performed on normal rats that had arterial catheters implanted to facilitate blood withdrawal without hemolysis. When ascorbate was administered by gavage to normal rats, blood ascorbate concentrations increased ~45% and were sustained for 240 min. When quercetin and ascorbate were administered together, quercetin significantly decreased ascorbate absorption ($p = 0.025$) (Fig. 9). These data suggest that quercetin may inhibit ascorbate intestinal transport *in vivo*.

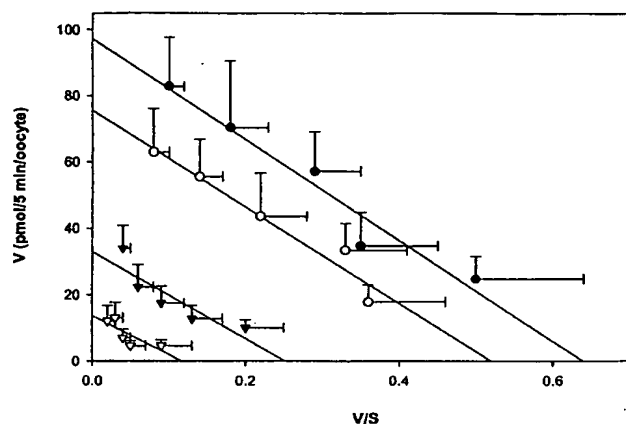


FIG. 6. Kinetics of quercetin inhibition of ascorbic acid transport in SVCT1(h) cRNA-injected oocytes. [^{14}C]Ascorbic acid 50, 100, 200, 400, or 800 μM was added with 10 μM (○), 20 μM (▼), or 50 μM (▽) quercetin or with 1% Me_2SO without quercetin (●). Oocytes were incubated for 5 min, washed, and analyzed for transported ascorbic acid as described under "Experimental Procedures." Kinetics are displayed using Eadie-Hofstee transformation. V/S, velocity/substrate concentration.

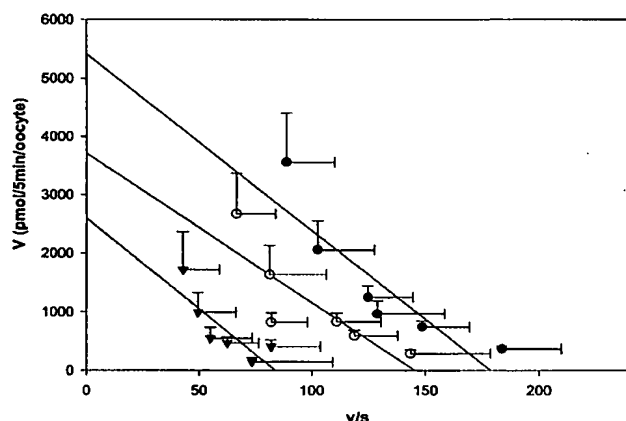


FIG. 7. Kinetics of quercetin inhibition of 2-deoxyglucose transport in GLUT2 cRNA-injected oocytes. [^3H]2-Deoxyglucose 2.5, 5.0, 7.5, 10, 20, or 40 mM was added with quercetin 10 μM (○) or 20 μM (●), or with 1% Me_2SO without quercetin (●). Oocytes were incubated for 5 min, washed, and analyzed for transported 2-deoxyglucose as described in "Experimental Procedures." Kinetics are displayed using Eadie-Hofstee transformation. V/S, velocity/substrate concentration.

DISCUSSION

The data in this paper show that flavonols and other flavonoids inhibited ascorbate transport by the intestinal transporter SVCT1(h). Inhibition of SVCT1(h) was shown in transiently transfected cells, stably transfected cells, and *X. laevis* oocytes. Using quercetin as a representative flavonol, inhibition occurred when ascorbate and quercetin were added simultaneously, was fully reversible, and was non-competitive. Non-competitive inhibition is consistent with flavonoid inhibition of ascorbate transport in myeloid cell lines, where the relevant ascorbate transporter was most likely SVCT2 rather than SVCT1 (11, 37). Flavonoid inhibition could not be explained by ascorbate oxidation, because quercetin did not decrease ascorbate stability. The relevance of the findings was strengthened by experimental results in normal rats, showing that ascorbate absorption was significantly decreased in animals given ascorbate and quercetin compared with animals given ascorbate alone.

Inhibition of ascorbate transport was dependent on flavonoid structure. The flavonol quercetin was an effective inhibitor,

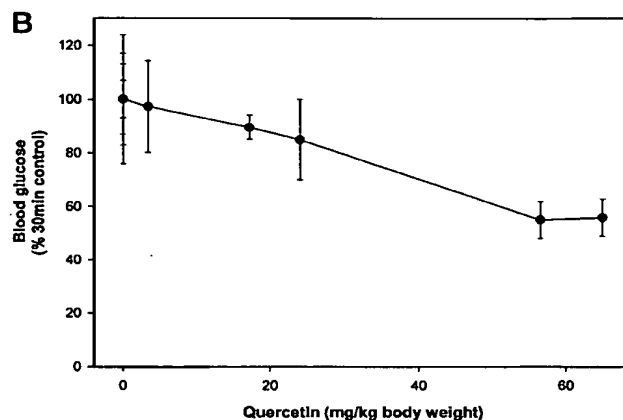
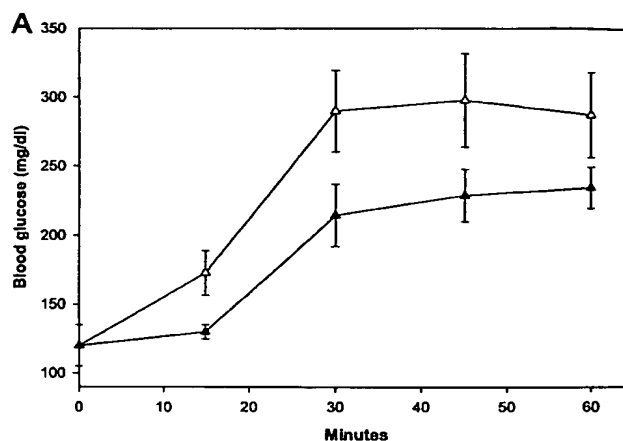


FIG. 8. Effects of quercetin on glucose absorption in diabetic rats. A, Zucker fa/fa rats fasted overnight were fed glucose solution (2 g/kg of body weight) with (▲) or without (△) quercetin (60 mg/kg of body weight). Blood glucose values (mg/dl) were determined by glucometer at the indicated times. Data from each rat in the presence and absence of quercetin were matched. $n = 5$, $p = 0.015$. B, Zucker fa/fa rats fasted overnight were fed glucose solution (2 g/kg of body weight) with or without quercetin 3–65 mg/kg/body weight. Blood glucose values from the 30-min time point were determined by glucometer and matched for each rat in the presence and absence of quercetin. Glucose values without quercetin (control) were normalized to 100%, and values in the presence of quercetin are expressed as % control. $n = 5$.

whereas its glycosylated precursor rutin was not. Myricetin and fisetin, other non-glycosylated flavonols, were also effective inhibitors. Flavonoids in foods are usually glycosylated (24). However, studies in human subjects with ileostomies show that quercetin glucosides are efficiently and possibly completely hydrolyzed to quercetin in the small intestine, most likely in the intestinal lumen (26, 38). Thus, quercetin and other flavonols should be available to inhibit ascorbate transport.

The data in this paper also indicate that quercetin inhibited intestinal glucose transport. With expression of the intestinal glucose transporter GLUT2 in *X. laevis* oocytes, quercetin inhibition of glucose transport occurred when substrate and inhibitor were added together, was reversible, and was non-competitive. The intestinal fructose transporter GLUT5 and the intestinal sodium-dependent glucose transporter SGLT1 were not inhibited by quercetin. When glucose and quercetin were co-administered orally in Zucker fa/fa rats, quercetin significantly blunted the hyperglycemia that occurred when glucose was administered alone. The most effective dose of

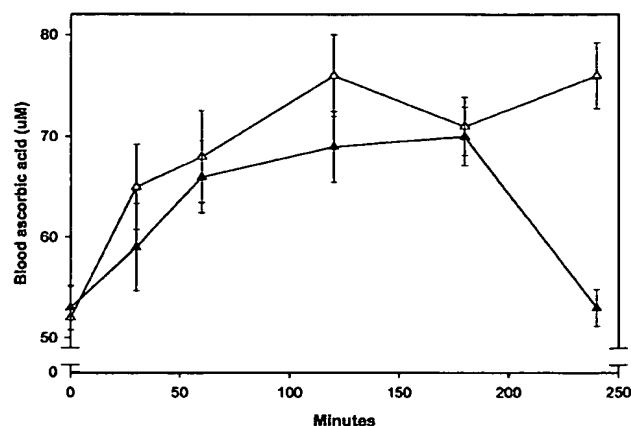


FIG. 9. Effects of quercetin on ascorbic acid absorption in normal rats. CD (Sprague-Dawley) rats after overnight fasting received by gavage ascorbic acid 60 mg/kg of body weight with (▲) or without (Δ) quercetin (15 mg/kg of body weight). Plasma ascorbic acid was determined by HPLC at the indicated times. Data from each rat in the presence and absence of quercetin were matched. $n = 10$, $p = 0.025$.

quercetin tested was ~60 mg/kg of body weight, equivalent to ~4 g for an adult human. However, the administered dose of glucose to achieve hyperglycemia in rats was 2 g/kg of body weight, approximately double the dose used in glucose tolerance testing in humans. Therefore, it is possible that the quercetin dose/kg of body weight needed to blunt hyperglycemia in humans might be lower than reported here in rats. In long term toxicity experiments in rats and hamsters, safe quercetin doses were at least 400 mg/kg/day and as high as 4000 mg/kg/day (39). Quercetin doses of 4 g were administered to humans orally without side effects (39). Based on the data, it is possible that the effects of quercetin might be relevant physiologically or for use in managing hyperglycemia (diabetes) (40).

The findings here raise new possibilities concerning flavonoids and their functions and may have physiological implications. Although flavonoid actions *in vivo* are unknown, they have been suggested to have broad antioxidant properties in humans (41). Flavonoids act as antioxidants at concentrations of 50 μM and above (42–46). However, peripheral venous and portal vein concentrations of flavonoids are usually 50-fold lower. After ingestion of flavonoid-rich foods, plasma flavonoid concentrations peak at ~1 μM , usually 1–2 h after ingestion (24, 25, 47). Fasting flavonoid concentrations are usually <0.5 μM . Most flavonoids have a half-life of 1–2 h, except quercetin. Flavonoid metabolites substantially increase the total antioxidant capacity of plasma, but the physiologic meaning of total antioxidant capacity is uncertain (24, 41). Although flavonoid metabolites could be systemic antioxidants, it is unlikely that flavonoids themselves act this way, implying that flavonoids may have other actions *in vivo*.

In cell lines, the flavonol myricetin inhibited ascorbate transport with K_i of 14 μM , and other flavonols had IC_{50} values of 16–40 μM (11). Although these concentrations are not realistic systemically, some are realistic as intestinal intraluminal concentrations. For example, dietary ingestion of quercetin and its glycone forms can be as much as 80 mg daily, and ingestion of total flavonoids can be as high as 130 mg daily (48). With an intestinal luminal distribution volume of 1 liter (24), glycone and aglycone quercetin concentrations may be as much as 200 μM . With deglycosylation, substantial concentrations of flavonoids may be present (24, 26).

We propose that novel functions of flavonoids may be to distribute nutrient absorption throughout small intestine or to

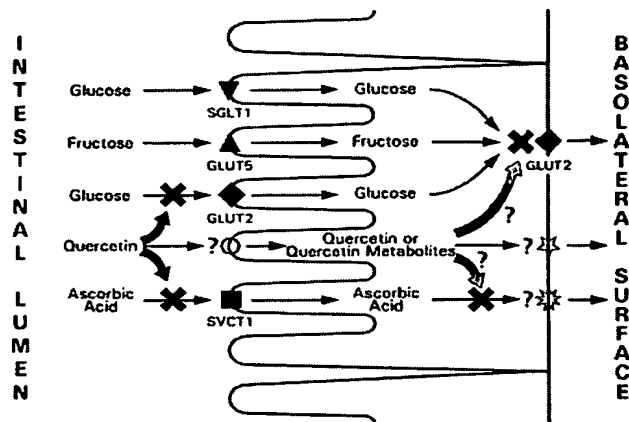


FIG. 10. Physiologic significance of flavonoid inhibition of ascorbic acid and glucose transport. As shown schematically, ascorbic acid and glucose absorption occurs from the left (intestinal lumen) to right (basolateral surface) side of an enterocyte. Quercetin inhibition of ascorbic acid transporter SVCT1 (■) and glucose transporter GLUT 2 (◆) is shown at the enterocyte luminal membrane (left side of figure). Quercetin has no effect on SGLT1 (▼) and GLUT5 (▲). The following are not certain (indicated by ?): mechanisms of quercetin entry and efflux; whether quercetin or its metabolites or both exit the enterocyte at the basolateral surface; mechanism of ascorbic acid efflux at the basolateral surface; whether quercetin or its metabolites within the enterocyte inhibit sugar or ascorbic acid efflux at the basolateral membrane.

frankly inhibit absorption. Flavonoids *in vivo* might possibly delay or inhibit ascorbate and glucose absorption by more than one pathway (Fig. 10). The data in this paper suggest that flavonoids could inhibit transport of ascorbate and glucose from the intestinal lumen into cells. For ascorbate, molecular mechanisms of absorption are incompletely characterized (49). Ascorbate must be transported across the luminal membrane of enterocytes but must also exit enterocytes on the basolateral surface to reach the portal venous system and the systemic circulation. Although SVCT1 is probably responsible for ascorbate transport into enterocytes from the intestinal lumen, it is unknown how ascorbate is translocated across the enterocyte basolateral membrane to enter the portal venous system. For glucose, emerging evidence implicates GLUT2 may be a major transporter of glucose from the luminal surface as well as from the basolateral surface (27, 33, 34). Thus, quercetin in the intestinal lumen could possibly inhibit both ascorbate and glucose influx into enterocytes. It is also possible that quercetin or its metabolites transported into enterocytes could inhibit efflux of intracellular ascorbate and glucose across the basolateral membrane. The mechanism of transport of quercetin itself is unknown, and the data here indicate that SVCT1(h) does not transport quercetin.

Flavonoid inhibition of enteric glucose and ascorbate transporters might result in broadening of peak post-absorptive plasma concentrations or frank inhibition of absorption, with clinical implications for each possibility. For ascorbate, broader and flatter peak post-absorptive plasma concentrations would result in less ascorbate excretion and higher distribution throughout body water. Frank inhibition of ascorbate absorption, as shown here in rats, would result in higher intestinal intraluminal concentrations, which could quench nitrosamines and other harmful oxidants (50–52). In either case, ascorbate bioavailability would change, with consequences for recommended dietary allowances of the vitamin (30, 53). For glucose, rapid glucose absorption in normal people might result in sharp fluctuations in blood glucose and resulting catecholaminergic hyperresponsiveness (54). In diabetics, rapid glucose ab-

sorption could exacerbate hyperglycemia. Decreasing or broadening glucose absorption can diminish these responses (40, 55). Animal data in this paper are consistent with flavonoid inhibition of glucose and ascorbic acid enteric transport *in vivo*. Studies of ascorbate absorption in animals are limited because of relatively low intestinal absorption of ascorbate (35, 36), lack of suitability of whole blood for rapid ascorbate analysis, and especially because of difficulty in obtaining plasma samples without hemolysis. The ascorbate absorption experiments described in this paper were made possible by obtaining blood samples from implanted carotid artery catheters, thereby minimizing hemolysis. In contrast to animal experiments, testing the effects of flavonoids on glucose and ascorbate absorption in humans should be straightforward (30, 56), and oral quercetin in humans at gram doses is safe (39, 57, 58). Based on the available evidence, it may be worthwhile to undertake clinical experiments to test the effects of flavonoids on ascorbate and glucose absorption.

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